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Protein Stabilization Explains the *gag* Requirement for Transformation of Lymphoid Cells by Abelson Murine Leukemia Virus

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The single protein encoded by Abelson murine leukemia virus is a fusion of sequence from the retroviral *gag* genes with the v-*abl* sequence. Deletion of most of the *gag* region from the transforming protein results in a virus capable of transforming fibroblasts but no longer capable of transforming lymphoid cells. Smaller deletions in *gag* reveal that p15 *gag* sequences are responsible for this effect, whereas deletion of p12 sequences had no effect on lymphoid transformation. In transformed fibroblasts, p15-deleted and normal proteins had similar activities and subcellular localization. When the p15-deleted genome was introduced into previously transformed lymphoid lines, its protein product exhibited a marked instability. The tyrosine-specific autophosphorylation activity per cell was less than 1/20th that of the nondeleted protein. Although pulse-labeling showed that the p15-deleted protein was synthesized efficiently, immunoblotting demonstrated that its steady-state level was less than 1/10th that of the nondeleted Abelson protein. The specific instability of the p15-deleted protein in lymphoid cells explains the requirement of these sequences for lymphoid but not fibroblast transformation.

Abelson Murine Leukemia virus (A-MuLV) is a replication-defective retrovirus capable of transforming fibroblast cells in vitro (28) and lymphoid cells both in vivo and in vitro (1, 26). The virus arose through recombination between Moloney-MuLV (M-MuLV), a replication-competent retrovirus, and c-*abl*, a gene normally expressed in many cells (7, 19, 31, 37, 38). The A-MuLV genome expresses a single gene product partially derived from each parent (23, 44). It contains 30 kilodaltons (kd) of M-MuLV *gag*-encoded sequence at its N terminus, followed by variable amounts of v-*abl*-derived sequence. The *gag* gene of M-MuLV encodes the viral core antigens in the form of a precursor protein, $Pr65^{gag}$, which contains p15, p12, p30, and p10, in that order. $Pr65^{gag}$ is cleaved into these products during maturation of the virion at the cell surface. The A-MuLV protein contains p15, p12, and a small part of p30 fused to the v-*abl*-encoded sequence.

The A-MuLV protein displays a protein-tyrosine kinase activity (42) which is apparently essential to the ability of the virus to transform cells (43). Mutations in the N terminus of v-*abl* abolish the kinase activity, as well as the transformation activity (20a; J. J.-Y. Wang and D. Baltimore, J. Biol. Chem., in press). The Rous sarcoma virus transforming gene product, $pp60^{src}$, as well as that of several other oncogenes (12, 13, 16) and growth factor receptor proteins (6, 11, 15, 34), has a protein-tyrosine kinase activity. Furthermore, these oncogenes and the epidermal growth factor receptor have extensive amino acid homology through a section of their genes which encodes the tyrosine kinase activity (3, 5).

There are many variants of A-MuLV containing different segments of v-*abl*. The largest variant, A-MuLV[P160], encodes a 160-kd protein, including 130 kd encoded by v-*abl* (9, 27). Only the N-terminal 45 kd of v-*abl* protein, containing the kinase domain and amino acid homology to $pp60^{src}$, is required for fibroblast transformation (21, 22). Although deletion of the C terminus of v-*abl* reduces the lymphoid transformation efficiency (27, 39), we show here that variants with the N-terminal 60 kd of v-*abl* protein retain the ability to transform lymphoid cells. For ease of reference, we call any v-*abl*-containing protein an Abelson protein.

Deletion of all of the *gag* sequences in A-MuLV, except for the first 34 amino acids, resulted in a virus which still transformed NIH/3T3 fibroblasts efficiently (21). Surprisingly, however, the deletion totally abolished the ability of the virus to transform lymphoid cells. Here, we report the effect of smaller deletions in *gag*. Deletion in p12 had no effect, whereas deletion in p15 abolished lymphoid-transforming activity. As we have not been able to detect any biochemical differences in the p15-deleted protein in transformed fibroblast cells, we introduced the p15-deleted virus into previously transformed lymphoid cells to study its properties in this specific cell type. We find a marked instability of the deleted protein specific to this cell type, resulting in drastically lower steady-state levels of the protein.

MATERIALS AND METHODS

Cells and viruses. Normal and A-MuLV-transformed NIH/3T3 cells were grown in Dulbecco modified Eagle's medium with 10% calf serum. A-MuLV titers were determined by focus formation on NIH/3T3 cells (28). Lymphoid cells were grown in RPMI 1640 medium supplemented with 10% fetal calf serum and 50 μM 2-mercaptoethanol. In vitro bone marrow transformation in agar and liquid have been described (25, 26). Transfections of NIH/3T3 cells were as described (41) and modified (21).

Infections of 70Z/3 and 2M3 cells were performed by resuspending 10^4 cells in 1.0 ml of a 1:1 dilution of a virus

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stock (with 8 μg of polybrene per ml) from either N2 or NΔp15-1 cells. The titers of these stocks were roughly 5×10^5 focus-forming units per ml. After 1.5 h of incubation with virus at 37°C the cells were single-cell cloned into microtiter wells. Single-cell clones were picked after 7 days from 96-well plates with less than 15 positive wells.

Cellular DNA and RNA. The preparation of total cellular DNA, restriction enzyme digestions, gel electrophoresis, and transfer to nitrocellulose (32) were all previously described (9). ^{32}P-labeled probes were prepared by nick translation (24).

Total cellular RNA was prepared by the guanidinium thiocyanate method (4), treated with glyoxal, and fractionated by electrophoresis through an agarose gel (18). The gel was transferred to nitrocellulose and hybridized with a nick-translated v-*abl*-specific probe.

Labeling, immunoprecipitation, and autophosphorylation. Cells were labeled with [^{35}S]methionine at 100 μCi/ml in methionine-free Dulbecco modified Eagle medium. Cells were extracted in phospholysis buffer (PLB; 10 mM sodium phosphate [pH 7.5], 0.1 M NaCl, 1% Triton X-100, 0.5% deoxycholate, 0.1% sodium dodecyl sulfate [SDS]), immunoprecipitated, and separated by electrophoresis through SDS-polyacrylamide gels as detailed elsewhere (44). Samples were normalized for trichloroacetic acid-precipitable radioactivity in each cell lysate before immunoprecipitation. A mouse monoclonal antibody against M-MuLV p12 was provided by Bruce Cheeseboro. A mouse monoclonal antibody to M-MuLV p15 was from Leslie Schiff-Maker and Naomi Rosenberg. R-anti-*abl*, a rabbit antiserum against v-*abl* protein expressed in bacteria, was provided by Jean Wang.

In vitro autophosphorylation reactions were essentially as described (42). Briefly, samples were normalized for cell number, extracted, and immunoprecipitated. The immune complexes were collected with 25 μl of 50% protein A-Sepharose (Pharmacia Fine Chemicals, Inc.) and washed three times in lysis buffer. The pellets were then washed two times in 50 mM Tris (pH 8) and resuspended in 50 μl of 20 mM Tris (pH 8)–10 mM $MnCl_2$–0.2 μl of 200 mCi of [γ-^{32}P]ATP (ICN East Pharmaceuticals). The autophosphorylation reaction was allowed to proceed for 10 min on ice. The pellet was then washed once with 1.0 ml of 50 mM Tris (pH 8) and resuspended in SDS-sample buffer (67 mM Tris [pH 6.8], 2% SDS, 10% glycerol, 5 mM EDTA, 20 mM dithiothreitol, bromophenol blue). The samples were boiled and fractionated by electrophoresis through an SDS- polyacrylamide gel in the standard fashion.

Immunoblotting. A-MuLV proteins were analyzed by immunoblotting essentially as described (33). Samples were normalized for cell number, extracted in 10 mM Tris (pH 7.5)–0.1 M NaCl–1% Triton X-100–0.5% deoxycholate–0.1% SDS for 10 min on ice, and clarified for 15 min at 13,000 × *g*. One-half volume of 3× SDS-sample buffer was added to the supernatants, boiled for 5 min, and fractionated by electrophoresis through a 7% SDS-polyacrylamide gel. The proteins were then transferred to nitrocelluose by using an electroblot apparatus (Bio-Rad Laboratories) at 275 mA for 2 h in 192 mM glycine–25 mM Tris-hydrochloride–20% (vol/vol) methanol. After transfer the filter was soaked for 1 h at room temperature in phosphate-buffered saline (PBS; pH 7.5) with 3% bovine serum albumin and 0.05% Tween 20 (blocking buffer), followed by 1 h at room temperature in blocking buffer containing a 1:1,000 dilution of R-anti-*abl*. The filter was then washed three times, 10 min each time, in PBS. As a second antibody, goat anti-rabbit immunoglobulin G conjugated to peroxidase (Cappel Laboratories) was used. The filter was incubated at room temperature for 30 min in blocking buffer containing a 1:1,000 dilution of the second antibody. The filter was again washed three times, 10 min each time. The specific signal was developed by placing the gel in 50 ml of water containing 50 mg of 3-3′-diaminobenzidine, 0.5 g of imidazole, and 50 μl of H_2O_2. When the signal was dark enough, the developer was stopped by washing the filter in PBS.

Cellular fractionation. Cells were normalized for number, washed once in PBS, resuspended in 1.0 ml of cold hypotonic Dounce buffer (5 mM KCl, 1 mM $MgCl_2$, 20 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid] [pH 7.0]), and allowed to swell for 10 min on ice. The samples were then Dounce homogenized 30 times and passed through a 25-gauge needle 10 times. This lysate was centrifuged at 1,000 × *g* for 5 min to remove nuclei and unlysed cells. The supernatant was centrifuged at 100,000 × *g* at 4°C for 30 min. The pellet (P100) was resuspended in 1× PLB, whereas 10× PLB was added to the supernatant (S100). Each fraction was immunoprecipitated and analyzed for autophosphorylation activity.

Plasmid constructions. All restriction enzymes (except *Eco*RI) and Bal 31 exonuclease were from New England Biolabs, Inc. *Eco*RI and the Klenow fragment of *Escherichia coli* polymerase I were from Boehringer Mannheim Biochemicals. Linker DNA and T4 DNA ligase were from Collaborative Research, Inc. Ampicillin-resistant colonies of *E. coli* HB101 were screened by rapid DNA preparation (14) and restriction digests. DNA was purified from agarose gels by the glass bead method (36). The various plasmids were constructed as follows.

(i) pABX2. pABX2 is a proviral plasmid containing a genome truncated at the 3′ end of v-*abl* at a *Xho*I site. This plasmid is the same as pABX (21), except that sequences at the 3′ end of the virus, corresponding to the *Hpa*I to *Cla*I sites of M-MuLV, have been deleted. This removes the possibility of the A-MuLV protein translating in frame with these sequences, which code for a part of p15E of M-MuLV. In addition the *Eco*RI site, at the junction of pBR322 and cellular flanking sequences, has been deleted.

(ii) pXΔp15. A deletion was made in pXΔp15 by placing an *Eco*RI 12-base-pair linker at two sites. The plasmid was digested with either *Bst*EII or *Nar*I. The cohesive ends were blunted with the Klenow fragment of *E. coli* polymerase I, and the *Eco*RI linkers were ligated on. Excess linkers were removed by *Eco*RI digestion. The DNA was also digested with a second enzyme and gel purified. The plasmid was reconstructed by three-part ligation of (i) *Hin*dIII-to-*Bst*EII (changed to an *Eco*RI site by the linker), (ii) *Nar*I (changed to an *Eco*RI site by the linker)-to-*Bgl*II, and (iii) *Bgl*II-to-*Hin*dIII fragments. This deletes amino acids 38 to 138 of *gag*, where the start codon methionine is counted as number 1. Three amino acids (Pro-Asn-Ser) are inserted at the deletion junction due to the linker DNA.

(iii) pXΔp12. pXΔp12 deletes amino acids 163 to 228 of *gag*, inserting three amino acids at the junction (Glu-Phe-Gly). This deletion was derived by using two previously constructed plasmids. One, pH3, contains an *Eco*RI linker insertion (Prywes et al., submitted for publication). The second contains a Bal 31 exonuclease-generated deletion with an *Eco*RI linker at the deletion junction. Piecing sections of these two plasmids together results in the deletion described above.

(iv) pABN2. pABN2 is exactly like pABX2, except for the end of its v-*abl* region. An *Eco*RI linker was placed at the

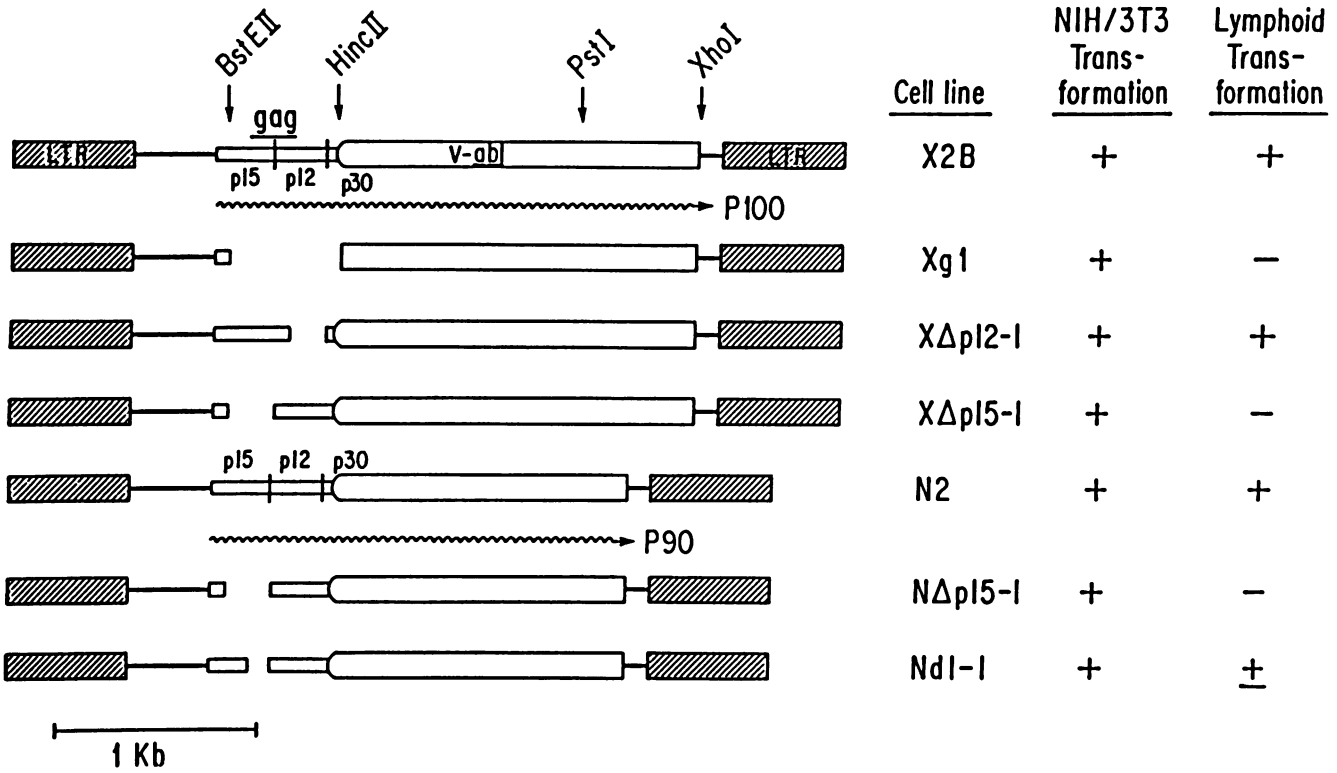

FIG. 1. Genomes of A-MuLV viruses used to test for the effect of *gag* deletions on lymphoid transformation. The deleted genomes of A-MuLV viruses, along with the names of the plasmids constructed to contain these genomes and the cell lines containing the genomes, are shown. The ability of the viruses to transform fibroblast and lymphoid cells is also indicated. All the plasmids transformed NIH/3T3 cells at an efficiency of ca. 500 foci per μg of DNA when cotransfected with M-MuLV DNA. See Table 1 for data on the efficiency of these viruses in lymphoid transformation.

*Nar*I site 377 bases 5′ of the *Xho*I site used in pABX2. The position that was the *Cla*I site of M-MuLV has also been changed to an *Eco*RI site. These two *Eco*RI sites were ligated together, deleting the 3′ 377 bases of v-*abl* present in pABX2.

(v) pNΔp15. pNΔp15 has the exact same deletion as pXΔp15 but in pABN2.

(vi) pNd1. pNd1 contains a deletion in pABN2 of amino acids 114 to 138 of *gag*, inserting two amino acids (Asn-Ser) at the junction. The plasmid was generated by treating pXΔp12 with *Eco*RI and then Bal 31 exonuclease. The reaction was terminated, the ends were blunted with the Klenow fragment of *E. coli* polymerase I, and *Eco*RI linkers were ligated on. An *Eco*RI-to-*Bgl*II fragment was gel purified and ligated to a gel-purified *Bgl*II-to-*Hin*dIII fragment of pABN2 and a gel-purified *Hin*dIII-to-*Eco*RI fragment of pNΔp15. This reconstructs the plasmid in a "pABN2" backbone. The deletion site was sequenced across by the method of Maxam and Gilbert (17), confirming that the coding region remains in frame.

(vii) pABGP. pABGP was generated from pABN2. pABN2 was digested partially with *Pst*I, and single-cut linear DNA was gel purified. This DNA was treated with the Klenow fragment to remove the single-stranded ends, and *Eco*RI linkers were ligated on and then digested with *Eco*RI to remove excess linkers and cut the plasmid at the end of v-*abl* in pABN2 (corresponding to what was the *Cla*I site of M-MuLV). The DNA was then ligated to close the circle and used to transform *E. coli*. Ampicillin-resistant colonies were screened for the *Eco*RI linker site at the desired *Pst*I site.

RESULTS

p15 and p12 deletions in *gag*. We have previously shown that deletion of all but the first 34 amino acids of *gag* in plasmid pXg1 results in a virus which transforms fibroblastic cells but has lost the ability to transform lymphoid cells (21). To localize the sequences in *gag* required for this effect, we made smaller deletions within *gag*. Because of a strong tendency of A-MuLV strains with a full C-terminal region to delete during passage of transformed cells (21, 45), plasmid pABX2, lacking 60 kd of C-terminal v-*abl* coding sequence, was the initial starting plasmid for these studies. Two deletions were made: pXΔp15, deleting amino acids 38 to 138 of *gag* (mainly p15 and 21 bases in p12), and pXΔp12, deleting amino acids 163 to 228 of *gag* (in p12 and a bit in p30; Fig. 1 and above). These plasmids were transfected into NIH/3T3 cells, along with helper virus M-MuLV DNA, to enhance the transfection efficiency (8). As expected, all three plasmids transformed NIH/3T3 cells at similar efficiencies of ca. 500 foci per μg of DNA.

To produce stocks of virus from the deleted plasmids, individual foci of transfected, transformed cells were picked and ascertained to contain the input viral genomes. For the parental plasmid, pABX2, we were able to isolate a cell line, X2B, containing only the expected genome as shown by *abl*-specific hybridization of cellular DNA cleaved by restriction enzymes in the long terminal repeats and fractionated by electrophoresis (9, 32) (data not shown). In addition, this cell line expressed the expected 100-kd Abelson protein. Unfortunately, the other plasmid-transformed cells con-

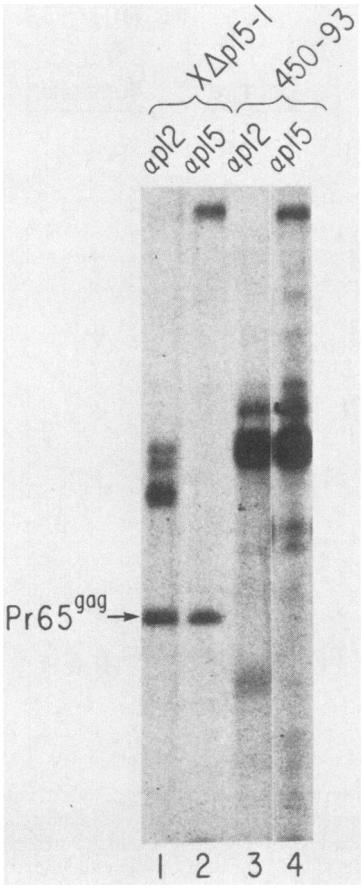

FIG. 2. The p15 determinants of the A-MuLV protein are present in a lymphoid transformant of XΔp15-1 virus. Cells were labeled with [^{35}S]methionine, extracted, and immunoprecipitated with either anti-M-MuLV p15 or anti-M-MuLV p12 monoclonal antiserum. The immunoprecipitates were analyzed by SDS–8% polyacrylamide electrophoresis as described in the text. The cell lines and antisera used are indicated above each lane. XΔp15-1 is a NIH/3T3 line transformed by pXΔp15. 490-3 is a lymphoid transformant of virus from XΔp15-1.

tained genomes that had undergone further, spontaneous deletions. Of three foci analyzed for pXΔp15 and for pXΔp12, none contained only the input genome. Either other forms of DNA were present along with the expected genome or only other forms were present (data not shown). We chose cell lines transformed by pXΔp15 and pXΔp12, called XΔp15-1 and XΔp12-1, respectively, which contained the expected genome, as well as other sizes of proviral DNA. In addition, by ^{35}S-metabolic labeling of these cells and immunoprecipitation, we confirmed that they contained Abelson proteins precipitable with specific *gag* antisera (either anti-p15 or anti-p12) but not by antisera directed against the deleted determinants (Fig. 2; data not shown).

Virus was harvested from each of the cell lines, and virus titers were determined by focus formation on NIH/3T3 cells. This virus was then tested for lymphoid transformation by the agar colony method (25). Bone marrow cells from adult mice were infected with 1 ml of undiluted virus and plated in agar. Lymphoid colonies were observable at 14 days postinfection. Transformation by A-MuLV[P160] is included as a control. Virus from X2B had a lower efficiency than A-MuLV[P160] but produced easily assayed transformants (Table 1). The efficiency of transformation by virus from XΔp12-1 was not reduced relative to X2B. Virus from XΔp15-1, however, had virtually no lymphoid transformation activity.

We did on occasion get one or two lymphoid colonies in an experiment with the p15-deleted virus of XΔp15-1. Cell lines were derived from these colonies, metabolically labeled with [^{35}S]methionine, and immunoprecipitated with either anti-p15 or anti-p12 monoclonal antiserum (one such line, 450-93, is shown in Fig. 2). Although XΔp15-1 contains an Abelson protein precipitable with anti-p12 but not anti-p15 antiserum (Fig. 2, lanes 1 and 2), the Abelson protein in 450-93 was precipitable with both antisera (lanes 3 and 4). Thus, the protein in 450-93 had regained p15 determinants, suggesting that these sequences are required for the virus to transform bone marrow cells. Reappearance of p15 sequences presumably occurred via recombination with the helper virus (which contains *gag* sequences), although recombination with endogenous retroviruses is a possibility. We rescued the recombinant virus from 450-93 cells and, as expected, it now transforms bone marrow cells at an efficiency comparable to that of X2B virus rather than XΔp15-1 virus.

Due to the spontaneous deletion problems encountered with pABX2, we extended these studies by using a slightly more truncated plasmid, pABN2, which lacks 377 3′ bases of v-*abl* present in pABX2 (Fig. 1). pABN2 is still capable of transforming fibroblasts efficiently upon transfection with M-MuLV, and to date we have not observed any v-*abl* deletions in cells transformed by it or any of its derivatives. Virus from one cell line, N2, derived from a pABN2-induced focus, could transform lymphoid cells somewhat more efficiently than virus from X2B, and therefore pABN2 appeared to be a good substrate to examine for the effect of other deletions.

We made two deletions in pABN2 (Fig. 1). The first, pNΔp15, was the same as that in pXΔp15 (deletion of amino acids 38 to 138); the second, pNd1, deleted amino acids 114 to 138. Both pNΔp15 and pNd1 transformed NIH/3T3 cells at a frequency comparable to that of pABN2. Two foci were picked, grown into cell lines, and shown to contain only the expected genomes. These cell lines, NΔp15-1, Nd1-1, and the parental N2, were metabolically labeled with [^{35}S]methionine and immunoprecipitated with a mouse anti-p12 monoclonal antibody. Proteins of the expected size, ca.

TABLE 1. In vitro bone marrow transformation by *gag*-deleted variants of Abelson MuLV

Cell line	Fibroblast titer (FFU/ml)[a]	No. of lymphoid colonies[b]: Per 10^6 cells	Per 10^5 FFU
Expt 1			
54/C12(P160)	3×10^5	145 ± 16	98 ± 11
X2B	5×10^5	5 ± 2	2.0 ± 0.4
XΔp15-1	1×10^6	1.0 ± 0.8	0.2 ± 0.2
XΔp12-1	1×10^5	7 ± 2	14 ± 4
Expt 2			
54/C12(P160)	1×10^5	64 ± 4	128 ± 8
N2	6×10^5	27 ± 3	9 ± 1
NΔp15-1	8×10^4	<0.13 ± <0.13	<0.31 ± <0.31
Nd1-1	1×10^5	0.4 ± 0.4	0.8 ± 0.8

[a] The virus titers were determined on NIH/3T3 cells. FFU, Focus-forming units.

[b] Nucleated bone marrow cells (2×10^6) were infected with 1.0 ml of virus and plated in agar. Lymphoid colonies appear at 10 to 14 days postinfection. The data are given as the average numbers of colonies ± the standard error of the mean.

90, 85, and 75 kd, were found in N2, Nd1-1, and NΔp15-1, respectively (Fig. 3). Virus from NΔp15-1, although it had a high titer on NIH/3T3 cells, had entirely lost its lymphoid-transforming activity and showed no reversion (Table 1). The efficiency of lymphoid transformation was also greatly reduced for virus from Nd1-1. Three of the rare transformants of Nd1-1 virus were analyzed to determine whether the low level of transformation was due to recombination. All had the same size of Abelson protein as in Nd1-1, indicating that they had not reverted (data not shown).

Expression of the p15-deleted virus in lymphoid cells. To examine why p15 sequences might be required for lymphoid transformation, we investigated the various properties of the deleted proteins expressed in transformed NIH/3T3 cells but did not find any significant differences from wild-type Abelson proteins. The *gag*-deleted proteins are still phosphorylated on tyrosine and elevate the in vivo phosphotyrosine level (21). Fractionation of cells into particulate and soluble fractions by 100,000 × *g* centrifugation failed to show a difference: approximately equivalent amounts of the Abelson protein were present in each fraction (data not shown).

Not finding any differences in the protein expressed in fibroblastic cells, we sought differences directly in lymphoid cells. Because the p15-deleted genomes do not transform lymphoid cells and because lymphoid cells do not grow extensively unless they are transformed, we could not directly produce a cell that contained only the deleted genome. We could, however, introduce the genomes nonselectively into a previously transformed cell line and screen for their uptake. To this end we used the chemically induced

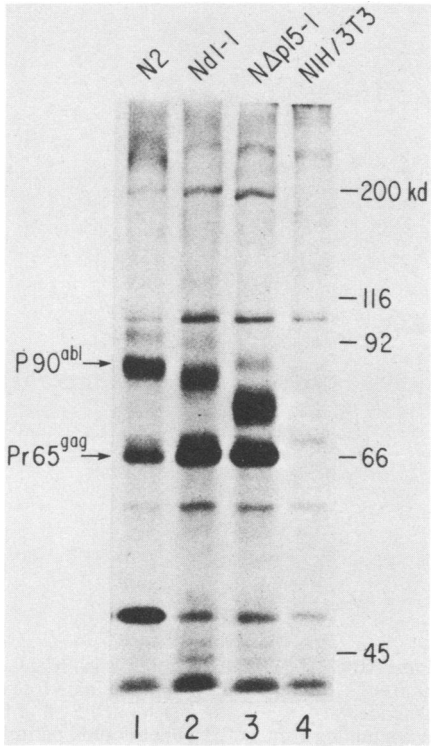

FIG. 3. Immunoprecipitation of A-MuLV proteins used to test for lymphoid transformation. Cells were labeled with [^{35}S]methionine for 1 h, extracted, immunoprecipitated with an anti-p12 monoclonal antibody, and analyzed by SDS–8% polyacrylamide electrophoresis in the standard fashion. The cell lines used are indicated above each lane, as are the positions of the specific immunoprecipitated proteins.

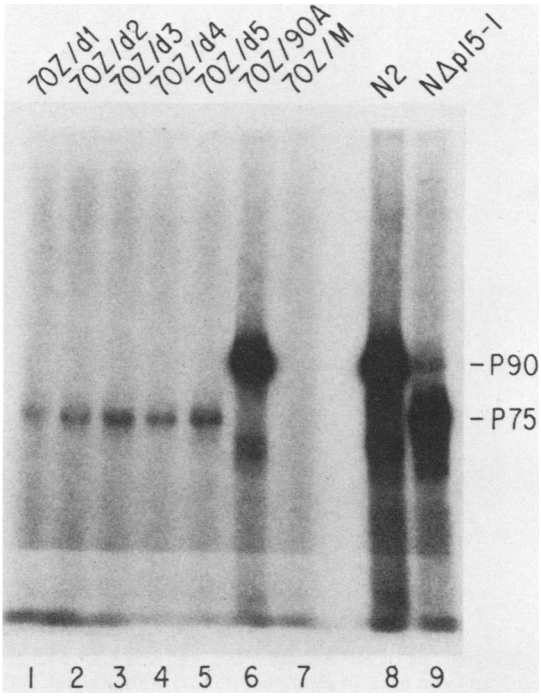

FIG. 4. In vitro kinase activity of A-MuLV proteins in 70Z/3 cells. Cells (10^6) were extracted, immunoprecipitated with an anti-p12 monoclonal antibody, and analyzed for in vitro autophosphorylation activity as described in the text. The cell lines used are indicated above each lane. 70Z/d1 through 70Z/d5 are lines derived from single-cell clones of 70Z/3 cells infected with the p15-deleted virus from NΔp15-1, whereas 70Z/90A is a single-cell clone infected with virus from N2 cells, and 70Z/M was infected with only M-MuLV. N2 and NΔp15-1 are transformed fibroblast lines containing P90 or the p15-deleted P75 A-MuLV proteins. The positions of the autophosphorylated A-MuLV proteins are marked.

lymphoma cell line 70Z/3 (20). It is slightly more advanced in its differentiated state than most A-MuLV-transformed lymphoid cell lines but has the advantage of lacking any known transforming virus. These cells were infected with virus stocks from either N2 or NΔp15-1 at a high multiplicity of infection and immediately cloned as single cells in microtiter dishes. The clonal cell lines were then screened for uptake of A-MuLV by testing immunoprecipitates of cells for autophosphorylation of an Abelson protein (42). Approximately 50% of the single-cell clones were positive in this assay. It was immediately evident that in five separate clones containing the p15-deleted genome, the autophosphorylation activity in the deleted P75 protein was 1/20th of that seen with the fully transforming P90 protein (Fig. 4, lanes 1 through 5). No autophosphorylation was evident in immunoprecipitates from a negative control, 70Z/M (lane 7), which was a single-cell clone of 70Z/3 infected only with M-MuLV. There was a negligible difference in autophosphorylation activity between the transformed fibroblast cells, N2 and NΔp15-1, containing the wild-type and p15-deleted genomes (lanes 8 and 9).

To determine whether the p15-deleted genome was being expressed efficiently in 70Z/3 cells, we made total cellular RNA from 70Z/90A (containing the undeleted virus), 70Z/d2 (containing the p15-deleted virus), and 70Z/M (with no A-MuLV genome) cells. The RNA was electrophoretically size fractionated and hybridized to a v-*abl*-specific probe. There were roughly equivalent amounts of A-MuLV RNA in

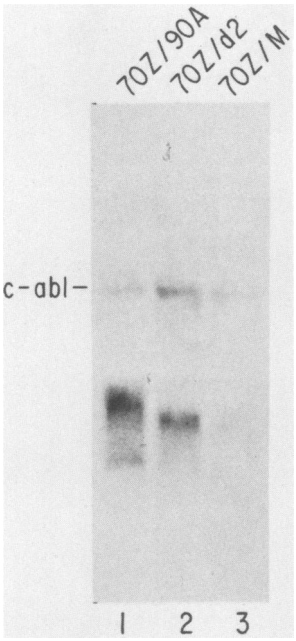

FIG. 5. Analysis of RNA from 70Z/3 cells. Total cellular RNA was prepared from each of the lines, treated with glyoxal, fractionated by electrophoresis through a 1% agarose gel, transferred to nitrocellulose, and hybridized with the v-*abl*-specific probe, pAB1sub9 (37), as described in the text. The cell lines used are indicated above each lane and described in the legend to Fig. 4. The position of endogenous c-*abl* RNA is marked.

70Z/90A and 70Z/d2 (Fig. 5), indicating that there is no block to expression of the p15-deleted genome in lymphoid cells. c-*abl* RNA was also evident in all three lines.

To examine the synthesis and steady-state levels of Abelson proteins in these cells, we performed two experiments. In one we pulse-labeled the cells with [^{35}S]methionine and immunoprecipitated the A-MuLV proteins. Both P90 and the p15-deleted P75 could be detected (Fig. 6). Although P75 was synthesized at a somewhat lower rate, the difference is not extensive enough to account for the differences in autophosphorylation activity.

We next used the immunoblotting technique to probe for the steady-state level of A-MuLV proteins. Lysates were prepared from each of the cell lines and electrophoresed through an SDS–7% polyacrylamide gel. The proteins were transferred to nitrocellulose and probed with a rabbit antibody (provided by Jean Wang) directed against v-*abl* protein purified from bacteria expressing the 5′ 1.2 kilobases of v-*abl*. Bound antibody was detected by using a second antibody stain of goat anti-rabbit immunoglobulin G conjugated to peroxidase. P75 and P90 were easily detectable in the fibroblastic cell lines NΔp15-1 and N2 (Fig. 7, lanes 2 and 3), although at a redued level in NΔp15-1. P90 was also clear in 70Z/90A (lane 8), but P75 was not detectable in 70Z/d2 (lane 7). We judge that the lower limit of sensitivity is less than 10% of the P90 level because we could detect P90 when its lysate was diluted with that of 70Z/M in a ratio of 1:10 (lane 9). Thus, although P75 is synthesized at appreciable rates, it does not accumulate to the steady-state levels of P90, presumably due to the specific instability of this protein in lymphoid cells.

Attempts to use pulse-chase experiments to demonstrate this instability directly have been confounded by background bands that comigrate with P75 and increase in intensity with chase time. Nonetheless, our estimate from several experiments is that the half-life of P90 in 70Z/90A is ca. 7 h, whereas the half-life of P75 in 70Z/d2 is ca. 1 h (data not shown).

To examine whether the instability was specific to 70Z/3 cells and to directly examine A-MuLV-transformed cells, we introduced the p15-deleted virus into a lymphoid cell line already transformed by a competent A-MuLV[P120] strain. This cell line, 2M3, is a nonproducer; because it does not contain M-MuLV, it has no superinfection barrier (44). A positive cell line, M8, was derived by superinfection with virus from NΔp15-1 and then tested by pulse-labeling and immunoblotting for synthesis and levels of the p15-deleted protein. P75 was synthesized in amounts similar to those of the endogenous P120, the Abelson transforming protein of 2M3 cells (Fig. 8). Immunoblotting showed undetectable P75, whereas P120 was clearly present (Fig. 7, lane 5). Just as in 70Z/3 cells, there appeared to be a marked instability of the p15-deleted protein in 2M3 cells.

One possible reason for the difference in stability between fibroblasts and lymphoid cells might be that the p15 sequences enable the protein to localize in a particular cellular

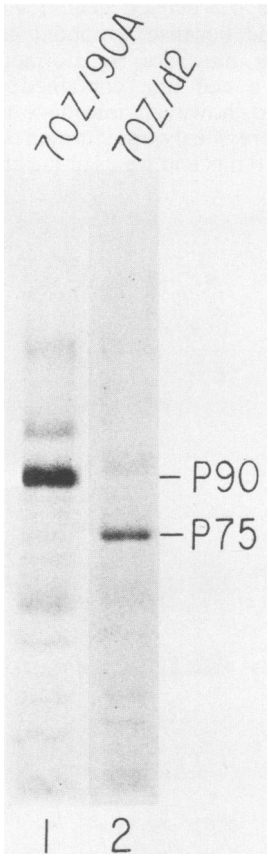

FIG. 6. Pulse-label of A-MuLV proteins in 70Z/3 cells. Cells were washed in PBS (pH 7.5), grown in methionine-free medium for 1 h and resuspended at 5×10^7 cells per ml in 0.5 ml of methionine-free medium containing 1 mCi of [^{35}S]methionine per ml for 10 min at 37°C. Cold PBS was added to stop the labeling. The cells were extracted and immunoprecipitated with R-anti-*abl*, a rabbit antiserum provided by Jean Wang, prepared against v-*abl* protein expressed in bacteria. The immunoprecipitates were analyzed by SDS–7% polyacrylamide electrophoresis as described in the text. The cell lines used are indicated above each lane. The positions of the precipitated A-MuLV proteins are indicated.

compartment. p15 is in fact the most hydrophobic of the *gag* proteins and will partition partially in membranes (2, 35). This is likely to be due in part to the fatty acid myristate, which is covalently linked to the N terminus of p15 (10, 30). The Abelson protein is also myristylated on its N terminus due to its p15 sequences (29). We found that the first 34 amino acids of *gag* present in the *gag*-deleted Abelson protein is sufficient for the protein to become myristylated (unpublished data). To test whether the p15-deleted protein, P75, fractionated differently than P90 in 70Z/3 cells, we fractionated 70Z/90A and 70Z/d2 cells and followed the A-MuLV proteins by autophosphorylation (Fig. 9). The cells were fractionated by 100,000 × *g* centrifugation into particulate (P100) and soluble (S100) fractions. These two fractions were then immunoprecipitated and analyzed for autophosphorylation activity. Although, again, the total autophosphorylation activity of P75 was less than 1/20th that of P90 (lanes 1 and 4), the activity that remained appeared to fractionate in a similar manner to that of P90, with a ratio of roughly 2:1 of P100 versus S100 activity (lanes 2, 3, 5, and 6).

Effect of v-*abl* deletions on lymphoid transformation. As described above, A-MuLV genomes deleted in v-*abl* back to the *Xho*I or *Nar*I sites in plasmids pABX2 and pABN2

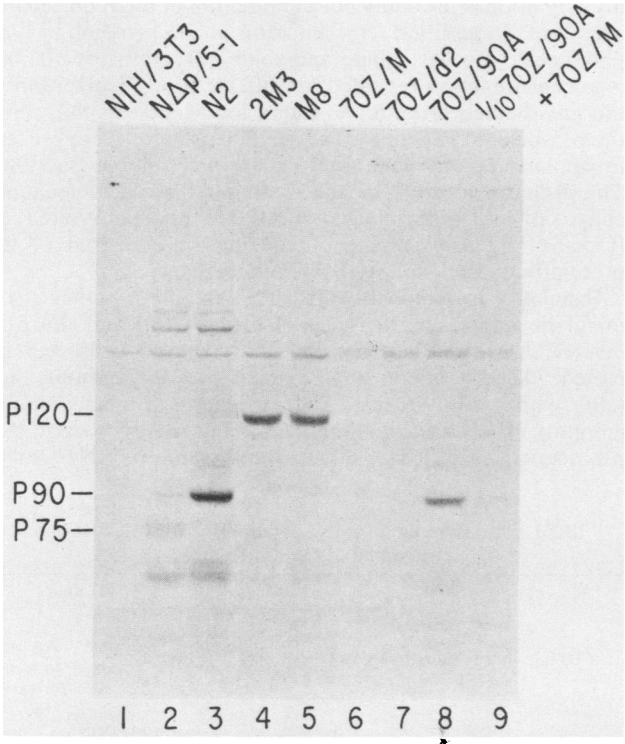

FIG. 7. Immunoblotting of A-MuLV proteins. Cells (5 × 10^5, lanes 1 through 3, or 1 × 10^6, lanes 4 through 9) were extracted, fractionated on an SDS–7% polyacrylamide gel, and transferred to nitrocellulose as described in the text. After a blocking step, the nitrocellulose filter was incubated with a 1:1,000 dilution of R-anti-*abl*, washed, and incubated with peroxidase conjugated goat anti-rabbit immunoglobulin G. After washing, the specific signal was developed with 3-3′-diaminobenzidene. These steps are all described in the text. The cell lines used are indicated above each lane and described in the legend to Fig. 4. In addition, 2M3 is an A-MuLV[P120]-transformed lymphoid line. M8 is a single-cell clone of 2M3 cells infected with p15-deleted virus. In lane 9, 1/10th of the lysate from 70Z/90A was mixed with 9/10ths of that from 70Z/M. The positions of A-MuLV proteins are indicated.

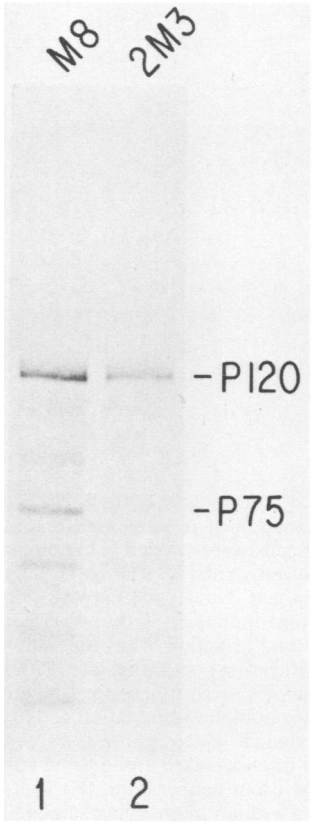

FIG. 8. Pulse-label of A-MuLV proteins in 2M3 cells. Cells were pulse-labeled for 10 min with [^{35}S]methionine and immunoprecipitated with R-anti-*abl* as described in the legend to Fig. 6. The immunoprecipitates were analyzed by SDS–7% polyacrylamide electrophoresis in the standard fashion. The cell lines used are indicated above each lane and described in the legend to Fig. 7. The positions of A-MuLV proteins are marked.

both retain the ability to transform lymphoid cells, although the efficiency is greatly reduced as compared with A-MuLV[P160] (Table 1). These two plasmids, however, have more of v-*abl* than is required for fibroblast transformation. We have previously found (21) that a *gag*-deleted plasmid, pAB-P, containing only the 5′ 1.2 kilobases of v-*abl*, will transform NIH/3T3 cells. As this plasmid was deleted in *gag*, we could not test whether this minimum transforming region was also sufficient for lymphoid transformation. For this reason we constructed a plasmid containing *gag* fused to the 5′ 1.2 kilobases of v-*abl*. This plasmid, pABGP, is exactly the same as pABN2, except that it extends to a *Pst*I site in v-*abl* rather than a *Nar*I site (see above for details of construction). The three plasmids pABGP, pABN2, and pABX2 were identical, except that they contain 1,221, 1,484, and 1,861 bases of v-*abl*, respectively. Plasmid pABGP transformed NIH/3T3 cells as expected, and we established a line, GP-2, from one of the foci. This line contains only the expected genome and the predicted 80-kd Abelson protein (data not shown).

We harvested virus from this line and tested it for lymphoid transformation. It was totally negative in an agar colony assay. We next tested the virus by a liquid transformation method in which bone marrow cells are infected and plated directly in liquid culture. This gives a greater sensitivity because a single transformation event can overtake the entire culture. The GP-2 virus was positive in this assay. Of

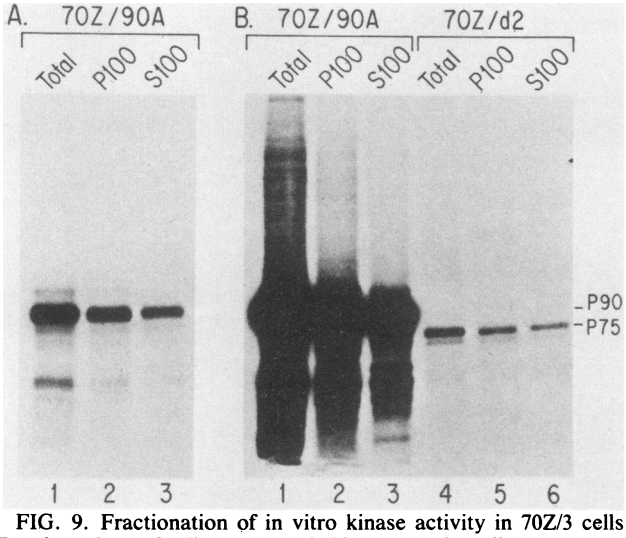

FIG. 9. Fractionation of in vitro kinase activity in 70Z/3 cells. Equal numbers of cells were swelled in hypotonic buffer and broken in a Dounce homogenizer and by passage through a 25-gauge needle as described in the text. Nuclei and unlysed cells were removed by 1,000 × *g* centrifugation. Half of the supernatant was saved and represented the "total" fraction. The other half was fractionated by 100,000 × *g* centrifugation into particulate (P100) and soluble (S100) fractions. The samples were immunoprecipitated with R-anti-*abl*, assayed for in vitro autophosphorylation activity, and analyzed by SDS–7% polyacrylamide electrophoresis as described in the text. The cell lines and fractions used are indicated above each lane. (A) 1 h of exposure at room temperature; (B) 16 h of exposure of the same gel at −70°C with an intensifying screen.

13 infected dishes, 3 became transformed. Virus derived from X2B and N2 cells transformed almost 100% of dishes infected (Table 2). The latency for the GP-2-infected plates was substantially longer than that for the other viruses. We checked two of the GP-2-transformed cultures for Abelson proteins and found in each a protein of the same size as in GP-2.

The low efficiency of the GP-2 virus may be a consequence of the low virus titer as tested on NIH/3T3 cells. GP-2 stocks were titered at 2×10^4 cells per ml, substantially lower than other viruses. The titer was even lower for another line derived from a focus transformed with pABGP. The two liquid cultures transformed with GP-2 virus and containing the expected protein showed clearly that this protein is capable of transforming lymphoid cells. We suspect that the GP-2 virus is intrinsically inefficient at lymphoid transformation, but the low titer of the stock makes definitive statements difficult.

DISCUSSION

We have examined why a previously described large deletion of *gag*-coding sequence from A-MuLV specifically abolished lymphoid cell transforming activity (21). We found that a deletion of only p15 sequences has the same dramatic effect, implying a localization of the crucial sequence to this subregion of *gag*. We further found that the p15 deletion labilized the protein in two different lymphoid cells, implying that the p15 sequences function to stabilize the protein specifically in lymphoid cells.

Localization to p15. The large *gag* deletion studied previously brought the N-terminal 34 amino acids of *gag* next to v-*abl*, and it could have been this specific juxtaposition of sequences that affected lymphoid-transforming activity rather than the loss of sequence due to deletion. This now seems unlikely because the p15 deletion described here left amino acids 1 to 37 (counting the initial methionine as number 1) and 139 to 236 of *gag* intact. Thus, the deletion left in place the 97 amino acids next to v-*abl* but had the same effect. The specificity of the effect to p15 sequences is indicated by the maintenance of lymphoid-transforming activity in a p12 deletion mutant. Furthermore, the small number of lymphoid transformants arising from infection of bone marrow cells with the p15-deleted virus could be accounted for by reacquisition of the p15 sequences, presumably via recombination with the helper virus. This again emphasizes the requirement of these p15 sequences for lymphoid transformation. Finally, a smaller deletion at the C terminus of p15 (amino acids 114 to 138 in pNd1) also caused a large reduction in lymphoid transformation, although there was some residual activity (Table 1).

In searching for an explanation of the role of p15 sequences, we noted that p15 is the most hydrophobic of the *gag* proteins and will partition into membranes (2, 35). In part, this is probably due to the myristyl group covalently linked to the N terminus of p15 (10). The A-MuLV protein is also myristylated on the end of its sequences (29), but we have found that the N-terminal 34 amino acids of *gag* still present in the *gag*-deleted protein are sufficient for signaling the myristylation (unpublished data), implying that signaling myristylation is probably not the function of the required p15 sequence. In addition, fractionation of the proteins in lymphoid cells into particulate and soluble fractions by 100,000 × *g* centrifugation revealed no difference in localization of the *gag*-deleted protein. As found for $P160^{gag\text{-}abl}$ (40), both the p15-deleted P75 and wild-type P90 proteins were present in similar amounts in the particulate and soluble fractions. The proteins not only localized similarly but are biochemically similar: in fibroblasts, both are phosphorylated on tyrosine and are capable of causing an elevation of the protein-bound phosphotyrosine level (21).

Instability in lymphoid cells. To examine whether there might be differences between A-MuLV[P90] and the p15-deleted A-MuLV[P75] specific to lymphoid cells, we infected 70Z/3, a chemically induced pre-B lymphoma line (20), with both viruses. The two viruses made similar amounts of RNA in this line, ruling out the possibility that p15 sequences seriously affect transcription or RNA stabil-

TABLE 2. Lymphoid transformation by A-MuLV variants truncated at their C terminus

Cell line	Fibroblast titer (FFU/ml)[a]	Agar		Liquid	
		Lymphoid colonies per 10^6 cells	Lymphoid colonies per 10^5 FFU	No. of cultures[c] transformed/ no. infected	Avg time[d] to transformation (days)
54/C12(P160)	1×10^5	64 ± 4	128 ± 8	9/9	11
X2B(P100)	5×10^5	5 ± 2	2.0 ± 0.4	7/8	15
N2(P90)	6×10^5	27 ± 3	9 ± 1	7/7	12
GP-2(P80)	2×10^4	<0.5 ± 0.5	<2 ± 2	3/13	23

[a] The virus titers were determined on NIH/3T3 cells. FFU, Focus-forming units.

[b] Nucleated bone marrow cells (2×10^6) were infected with 1.0 ml of virus and plated in agar. Lymphoid colonies appear at 10 to 14 days postinfection. The data are given as the average number of colonies ± the standard error of the mean.

[c] Nucleated bone marrow cells (2×10^6) were infected with 1.0 ml of virus and plated in liquid.

[d] The average time required to observe lymphoid transformants overtaking the culture is given. Nontransformed cultures were held 35 to 40 days.

ity. Pulse-labeling of the 70Z/3 lines showed that both proteins were synthesized efficiently. There was, however, a striking difference in autophosphorylation activity of P90 compared with P75. This was explained when electrophoretic fractionation, and staining with peroxidase-coupled antibody showed that the steady-state amount of P90 was much higher than that of P75 in the 70Z/3 cell lines. By contrast, in transformed fibroblast lines, P90 and P75 had more similar amounts and autophosphorylation activities. In an A-MuLV-induced lymphoid cell line, 2M3, the same instability of the p15-deleted protein as in 70Z/3 cells was evident. The results in these two cell lines show that in lymphoid cells there is a specific instability of the p15-deleted Abelson protein, which presumably explains the inability of p15-deleted Abelson viruses to transform bone marrow cells.

Importance of *gag* sequence in A-MuLV. The large deletion in *gag* (21) and now the smaller deletions within p15 have shown that some of the *gag* sequences in A-MuLV are integral parts of this transforming virus. These sequences are required to stabilize the transforming protein in lymphoid cells. If the recombination event to form the virus had occurred at amino acid 34 of p15, rather than within p30, our data clearly indicate that the virus would not be transforming and would never have been detected. (Remember that in animals A-MuLV transforms lymphoid cells but not fibroblasts.) Whether the inclusion of *gag* sequences (or some other foreign sequence) is a necessary event in the modification of the nononcogenic proto-oncogene, c-*abl*, to form the v-*abl* oncogene is a more difficult question to answer. Because the N terminus of c-*abl* is missing from A-MuLV (37, 38), we do not know whether the *gag* sequence is replacing the normal function of the c-*abl* N-terminal sequence or whether *gag* is imparting new properties to the *abl* protein. B. Mathey-Prevot and D. Baltimore (unpublished data) have shown that the N terminus of $pp60^{src}$ will replace the *gag* function in A-MuLV lymphoid transformation, showing that *gag* is not unique in providing the stabilizing activity.

Although the deletion of the c-*abl* N terminus and its replacement by *gag* sequence may be the events that activate c-*abl* to become the v-*abl* oncogene, there are other differences between c-*abl* and v-*abl*. For one thing, there are many point mutations in v-*abl* relative to c-*abl* which may be important (38). Also, due to the strong retrovirus promoter, there is an elevated expression of the Abelson transforming protein over the c-*abl* product of ca. 14-fold (37). Any or all of these alterations could be responsible for making c-*abl* into an oncogene.

The reason p15 sequences stabilize the A-MuLV protein specifically in lymphoid cells is not clear. Because p15 is hydrophobic, we suspected that it might affect the localization of the protein to the membrane. Crude fractionation by $100,000 \times g$ centrifugation, however, suggests that, at least for the autophosphorylation activity that remains, there is no difference in localization. Perhaps there are more subtle differences in localization, such as localization to specific organelles or complexes which might stabilize the protein. A simpler hypothesis is that deletion in p15 may simply destabilize the protein due to changes in secondary structure which make it more accessible to proteases. For this to be true, either these proteases must be specific to lymphoid cells or the change in secondary structure must not be as pronounced in fibroblasts.

A final point made by these experiments is that the minimum transforming region of v-*abl* is the same for fibroblasts and lymphoid cells. The derivation of lymphoid clones transformed by the GP-2 virus demonstrates this point; it has a v-*abl* region with as small a deletion of v-*abl* sequence as any that will transform fibroblasts (20a, 21). The GP-2 virus is inefficient at transformation but also, for unknown reasons, has a low titer. Previous data showed that C-terminal v-*abl* sequences play a quantitative but not qualitative role in A-MuLV transformation (21, 27); perhaps GP-2 shows this effect most dramatically, but until higher-titer stocks are available this point will remain unclear.

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